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Developmental Morphology of Young Gametophytes of *Botrychium microphyllum* in Axenic Culture

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Abstract: To clarify the origin of the apical meristem in the fern gametophyte, we examined the early development of young gametophytes of *Botrychium microphyllum* (Sahashi) K. Iwats. (Botrychiaceae) in axenic culture. At the earliest stage, the gametophyte was completely spherical or somewhat elongated with no apical meristem. The apical meristem was later formed at the periphery of the gametophyte apex, and thereafter gametophytes became arched owing to the apical meristem activity. In conclusion, the young gametophyte was composed of two parts: (1) the early spherical mass and (2) the later formed apical conical part with an apical meristem.

Introduction

Gametophytes of ferns are usually terrestrial, chlorophyllous, and heart- or ribbon shape, with a thickness of one cell-layer¹⁾. In contrast, subterranean gametophytes of some fern families such as Psilotaceae and Botrychiaceae are mycorrhizic and non-chlorophyllous, with cylindrical, globular, or conical shapes^{2, 3)}. Recent phylogenetic analyses elucidated that Psilotaceae (whisk ferns) and Botrychiaceae (Ophioglossoid ferns), which are sisters, are nested to the base of the ferns (monilophytes)⁴⁾. Microphyllous lycophytes, a group sister to euphyllophytes (ferns and seed plants), similarly have subterranean mycorrhizic gametophytes. On the other hand, gametophytes of primitive ferns such as marattioid ferns, horsetails, osmundaceous ferns, are chlorophyllous (non mycorrhizic) and retain the spherical mass at early developmental stages⁵⁾. This is in contrast to ordinary heart shaped gametophytes, which show a thickness of one cell-layer from the beginning of development. These facts imply that fern gametophytes may have changed shape from the massive to the one cell-layer thick, associated with loss of endophytic fungi through evolution. To elucidate this evolutionary scenario of gametophytes,

comparative development focusing on meristem behaviors between globular-, and heart-shaped gametophytes is necessary.

Gametophyte development is attributable to apical meristems, which are formed at an early stage of development. Heart shaped gametophytes, a common type of fern gametophyte, have the apical meristem with a single apical cell at inception, but the apical cell is soon replaced by plural apical initials¹⁾. Mycorrhizic massive gametophytes of Botrychiaceae have similarly apical meristems⁶⁻⁹⁾, but it has not been clarified yet how and where the apical meristem is formed. Until the early 1900s, mycorrhizic gametophytes collected in the field had been used for research^{6, 7)}, but the use of sterile cultures has enhanced studies of gametophytes with endophytic fungi⁸⁻¹⁰⁾. Whittier and Thomas¹¹⁾ succeeded to form sexually mature gametophytes with sporophytes of *Botrychium jenmanii* in axenic culture. The cultured gametophytes appear to be same structures as those from the field, with only differences in presence or absence of endophytic fungi. To clarify the initiation of the apical meristem, we examined the early development of *Botrychium microphyllum* gametophytes in axenic culture.

Materials and methods

Spores of *Botrychium microphyllum* (Sahashi) K. Iwats.

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were generously given by Prof. Norio Sahashi, Toho University. The spores were sterilized with 0.5% sodium hypochlorite solution for 5 min, washed with sterilized water several times, and cultured in the Whittier and Thomas's nutrient medium¹¹⁾ at $24 \pm 1^\circ\text{C}$ in darkness. The nutrient medium was composed of FeEDTA, minor element solution, 0.2% glucose, and 0.8% agar in pH of 6.1¹¹⁾. After 14 months in culture, gametophytes at various developmental stages were obtained, including just germinated spores. The gametophytes were fixed with FAA, embedded in Historesin (Leica, Heiderberg) or Technovit 7100 (Heraeus Kulzer, Wehrheim), sectioned at a thickness of $2\text{--}3\ \mu\text{m}$ and stained with safranin, toluidine blue, and orange G¹²⁾. For SEM observation, well-grown gametophytes fixed with FAA were dehydrated with ethanol series, critical point dried, coated with platinum-palladium, and observed with SEM (3D microscope Real Surface View VE-8800, KEYENCE, Osaka).

Results and discussion

The spore coat opened at the triradiate ridge. The first cell division in the spores was nearly transverse to its polar axis, producing a distal cell (away from the triradiate ridge) and a proximal cell (near the triradiate ridge) (Fig. 1). After this stage, both cells underwent cell divisions to form a spherical mass consisting of several cells (Fig. 2), and further a somewhat elongated globular gametophyte (Fig. 3). Whittier¹³⁾ reported that in four species of *Botrychium* the proximal cell at the

two-cell stage may divide to form 3 or 4 cells as the gametophyte matures, while the distal cell gives rise to the massive gametophyte through frequent cell divisions. Our examination on *B. microphyllum* showed that in many young gametophytes a small number of densely stained cells with spore coats were attached to the main mass of equally-sized cells (Figs. 4, 5). These distinct cells with spore coats may correspond to Whittier's "proximal cell derivatives", suggesting that young gametophytes were mostly derived from one of the two cells formed just after spore germination (see Fig. 1).

More grown elongated gametophytes consisted of equally-sized cells with many starch-grains, showing no apical meristem formed yet (Fig. 6). The spore coat was still attached to the proximal end. Thereafter, the distal and proximal ends of the gametophytes are referred to as the apical and the basal ends of the gametophytes, respectively. It is noted that some surface cells near the basal end became enlarged to protrude from the surface, though their function is unknown (Figs. 5, 6). These protruded surface cells were still found even in more developed gametophytes (Fig. 9). In addition, rhizoidal hairs began to be formed through out the entire body surface.

In more grown gametophytes, an apical meristem consisting of small densely stained cells arose from the side, not the central, region of the gametophyte apex (Fig. 6). Owing to the apical meristem, the apical portion of the gametophyte became tapered with a conical shape, bending at right angle to the apical-basal axis

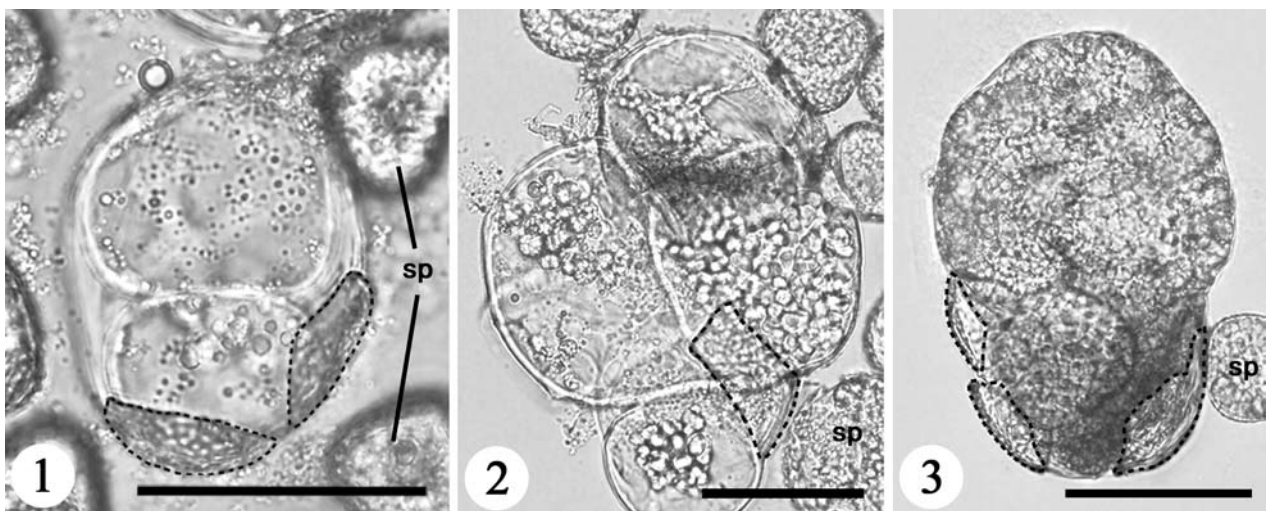


Fig. 1–3. Just germinated spore (1) and gametophytes at early developmental stages (2, 3). Dashed lines outline spore coats attached to the gametophytes. 1. Two-cell stage. 2. Nearly five-cell stage. 3. Further grown ovoid gametophyte. sp, ungerminated spore. Scale bars = $50\ \mu\text{m}$.

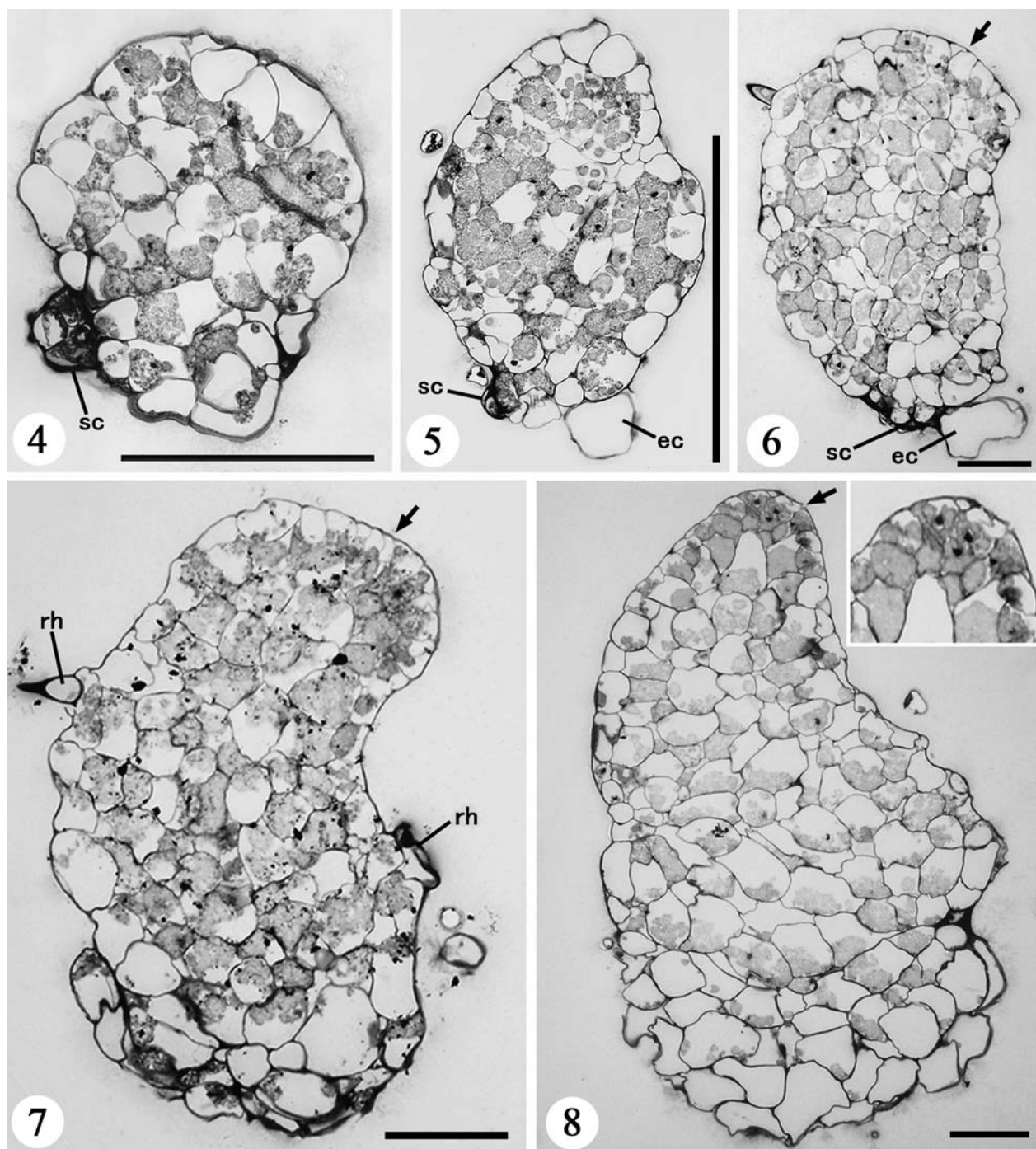


Fig. 4-8. Median longitudinal sections of growing gametophytes. 4, 5. Ovoid and somewhat elongate gametophytes. Note that ovoid mass of large cells (gametophyte proper) is attached by a group of densely stained cells with spore coat. The apical meristem has not been established yet. 6. More developed gametophyte with just initiating apical meristem (arrow). 7, 8. Slightly bending gametophytes with apical meristems (arrows). Inset in 8 is a close up of the apical meristem. Some basal cells are enlarged with very little starch grains. ec, enlarged epidermal cell; rh, rhizoid; sc, spore coat remnant. Scale bars=100 μ m.

(Figs. 7, 8). Bending apical portions became more apparent in further grown gametophytes (Figs. 9, 11). In extreme cases, the apical portions were arched

toward the base of the gametophyte (Fig. 12). Such bending of gametophyte is unique in shape differing from other *Botrychium* gametophytes so far examined:

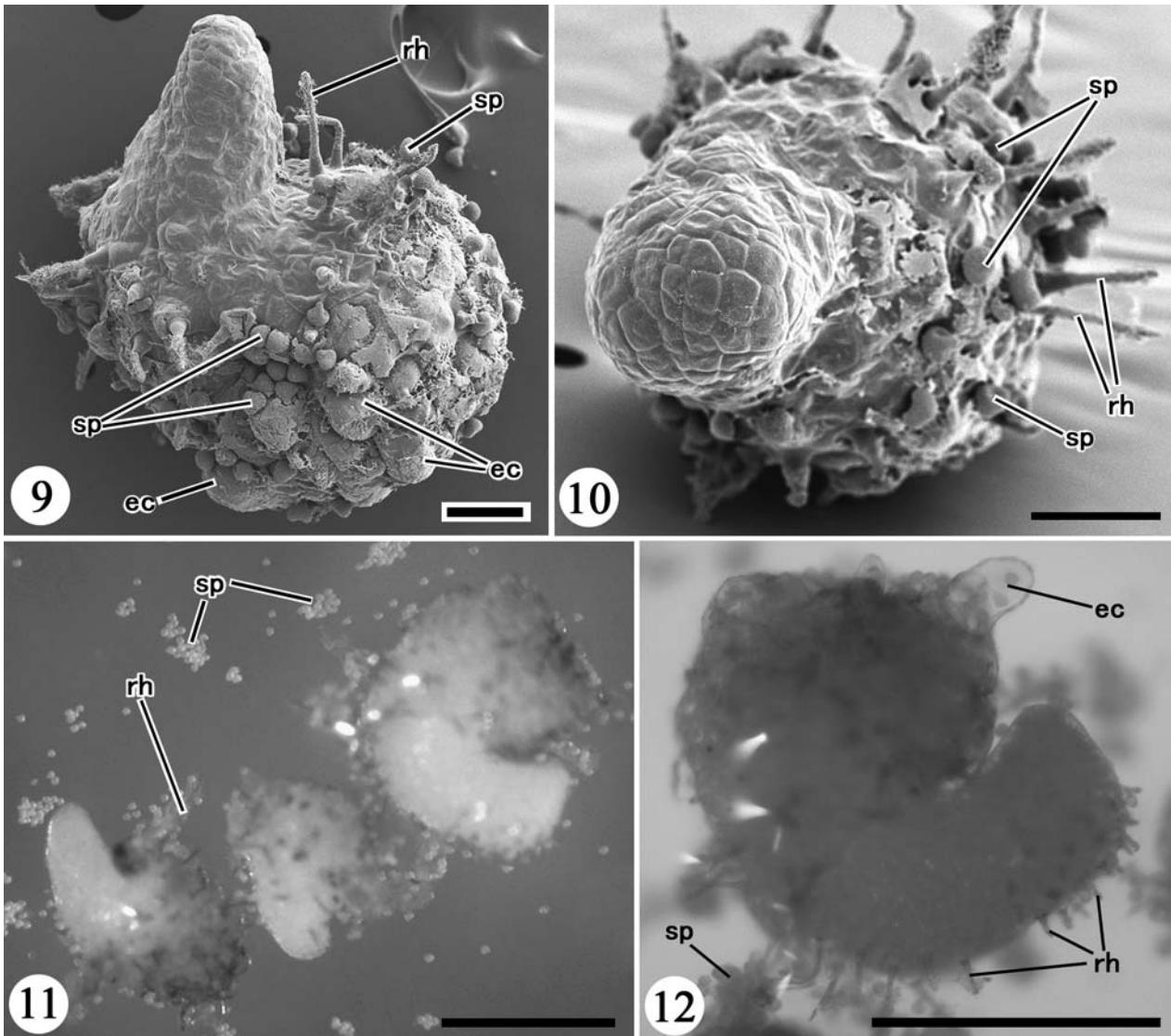


Fig. 9 – 12. Further grown bending gametophytes. 9, 10. SEM images of a gametophyte. Gametophyte is composed of basal massive part and apical conical part. Close up of apical region in 9 is shown in 10. The apical meristem has no apparent apical cell. 11. Microscope image of three gametophytes of different sizes. 12. Microscope image of the largest gametophyte in 11, through transmitted light. ec, enlarged epidermal cell; rh, rhizoid; sp, ungerminated spores. Scale bars = 100 μ m for 9, 10, 1 mm for 11, 12.

flattened shape¹⁴), bean-, strap-, or spherical-shape¹⁵), globular of approximately hemispherical¹⁶), or ovate shape¹⁷).

The apical meristem consists of a small number of meristematic cells with no apparent apical cell (Figs. 8, 10). The apical cell was reported only for *B. multifidum*¹⁷), and not recognized for other *Botrychium* species^{8, 13, 15}). Absence of the apical cell in young gametophytes is in marked contrast to the presence of it for *Ophioglossum* gametophytes of cylindrical shape^{9, 18, 19}).

Young gametophytes have brownish colored basal

cells (Fig. 3). SEM images of grown gametophytes showed that the surface of the basal spherical half was covered with some substances and ungerminated spores, while the apical half had a smooth surface with exposed epidermal layer (Figs. 9, 10). These brownish and coating substances are probably equivalent to a thin coating of mucilage over the proximal cell wall just after spore germination for *B. dissectum* in hydroculture²⁰), and mucilage sheath of rhizoids for *B. lunarioides*²¹). It seems likely that the mucilage substances were continuously secreted during early developmental stages.

In conclusion, early development of *Botrychium*

microphyllum gametophytes consisted of two different phases: (1) no meristem phase in which a mass of equally-sized cells was formed, and (2) the apical meristem phase by which the conical portion of gametophytes were added to the early sphere. The apical meristem had no recognizable apical cell.

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イブリハナワラビの無菌培養配偶体の形態形成

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要旨 シダ植物ハナヤスリ科の配偶体は地中生で、葉緑体をもたず光合成を行うことができないため、自然界では共生菌から栄養を得て時間をかけて成長する。したがって発生の初期過程の研究が大きく遅れている。本研究では同科ハナワラビ属のイブリハナワラビの胞子を無菌培養することにより、人工的に配偶体を発生させ、その初期発生過程を明らかにした。初期配偶体は球状で、頂端分裂組織は存在しなかった。やがて卵形になった配偶体の先端部に頂端分裂組織が形成された。成長途中の配偶体は2つの部分、(1) 胞子由来の初期球状部、と (2) 頂端分裂組織の働きによってつくられた円錐形部、から構成される。ハナヤスリ属の配偶体でその存在が報告されている頂端細胞は、本種の頂端分裂組織には観察されなかった。頂端分裂組織の位置は中央ではなく端にずれているため、配偶体は横方向に曲がりながら成長していった。このような形態の配偶体は初めての報告である。

